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# Prevalence of high-risk human papillomavirus infection and cancer gene mutations in nonmalignant tonsils

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## ABSTRACT

**Objectives:** To analyze the prevalence of high-risk HPV (human papillomavirus) and genetic alterations in nonmalignant tonsils.

**Methods:** We collected benign fresh tonsillar tissue specimens from 477 patients undergoing tonsillectomy because of chronic tonsillitis or tonsillar hypertrophy in 2012 (Group A, n = 237) and in 2015 (Group B, n = 240). Luminex xMAP technique served to detect E6/E7 DNA from 16 different high-risk HPV types. Tonsillar DNA and peripheral blood leukocyte DNA from the infected individuals were analyzed using Nimblegen SeqCap EZ Comprehensive Cancer Design panel. The panel targets 578 different genes that are relevant in carcinogenesis. HPV negative tonsillar specimens from age- and gender matched individuals were used as controls. All specimens harboring high-risk HPV were analyzed using fluorescence in situ hybridization (FISH).

**Results:** Five of 477 (1.0%) patients tested positive for the following HPV types: HPV16 (two cases), HPV52 (one case), HPV66 (one case), HPV52 and HPV68 (coinfection, one case). FISH analyses showed that the appearance of HPV in specimens infected with HPV 16 was episomal. Benign tonsils infected with high-risk HPV harbored mutations in *EP300*, *NF1*, *PIK3CA*, and *RB1* which are considered relevant in the development of HPV-associated head and neck squamous cell carcinoma (SCC).

**Conclusions:** The prevalence of high-risk HPV in nonmalignant tonsils is low. High-risk HPV positive tonsils harbored mutations in genes that are commonly altered in HPV-associated head and neck SCC. The role of these mutations in tonsillar carcinogenesis is an interesting target for future research.

## Introduction

Head and neck squamous cell carcinoma (SCC) accounts for approximately 600,000 new cancer cases each year [1]. The main risk factors are tobacco and alcohol use. In many developed countries, however, the majority of newly diagnosed tonsillar SCCs are associated with high-risk human papillomavirus (HPV) infection, predominantly HPV type 16 [2,3]. Compared to individuals with HPV negative tonsillar SCC, those with HPV positive disease have better prognosis, and they are often younger with no significant history of tobacco or alcohol use [4–6].

It is unclear why some individuals with high-risk HPV infections develop malignant disease, while others are able to clear the infection. Considering the significant role of HPV in tonsillar SCC, it is interesting that HPV infections in non-malignant tonsils are extremely rare. Benign tonsillar specimens collected from nearly 4000 tonsillectomy patients in the UK were all HPV negative [7]. Because HPV prevalence in non-malignant tonsils is low, and the proportion of HPV positive malignant tonsils is high, a presumption has been raised that nonmalignant tonsils harboring high-risk HPV may have a high risk of progression to cancer. Cytological brush samples and excisional biopsies are used to detect precancerous lesion from the uterine cervix [8]. A similar precursor

Abbreviations: FISH, fluorescence in situ hybridization; HPV, human papillomavirus; IHC, immunohistochemistry; SCC, squamous cell carcinoma; UTR, untranslated region

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lesion, that could be detected and treated before cancer fully develops, has not been characterized for HPV-associated tonsillar SCC.

HPV has two important oncogenes, E6 and E7, whose expression may increase upon integration of HPV into chromosomal DNA. In HPV-associated carcinogenesis viral E6 and E7 proteins inactivate the function of cellular p53 and retinoblastoma (Rb) tumor suppressors, respectively, which leads to dysregulation of the cell cycle, inhibition of apoptosis, and eventually genetic instability [9]. Molecular genetic analyses of HPV positive and HPV negative head and neck SCCs have revealed that both chromosomal and mutational profiles in these two distinct cancer types are different. Compared to tobacco associated head and neck SCC, the number of mutations in HPV positive SCC is typically lower. HPV negative head and neck SCCs harbor mutations in *TP53*, unlike HPV positive SCCs where *TP53* gene is wild-type but viral E6 protein degrades the protein product [10–15]. Massive parallel sequencing of 120 matched pairs of head and neck tumor/normal samples showed enrichment of mutations in *PIK3CA*, *MLL3*, *DDX3X*, *FGFR2/3*, *NOTCH1*, *NF1*, *KRAS*, and *FBXW7* in HPV positive SCC [16]. In addition to these genes, previous studies have detected alterations in *ASXL3*, *EP300*, *ZNF750*, *TTN*, and *SYNE1* [12,13,15,17]. Analysis of HPV negative head and neck SCC has shown enrichment for mutations in a different set of genes: *TP3*, *FAT1*, *CDKN2A*, *TTN*, *SYNE1*, *NOTCH1*, *PIK3CA*, and *NSD1* [18].

Systematic detection of mutations in cancer-relevant genes has become feasible due to development of cancer sequencing gene panels [19]. When considering preventive measures or targeted molecular cancer therapies for HPV-associated disease, understanding the molecular events preceding carcinogenesis is crucial. Defining which age groups have the highest prevalence of tonsillar HPV infection helps to understand the sexual and non-sexual routes of HPV transmission as well as the natural course of the infection. The specific morphological changes and mutations preceding HPV-associated SCC are poorly understood. Finding a precursor for tonsillar HPV-associated cancer would help in the early detection of cancer, and in identifying individuals with an increased risk of malignancy, who would possibly benefit from preventive measures such as HPV-vaccination.

The aim of this study was to determine the prevalence of high-risk HPV in nonmalignant tonsils, and to compare the prevalence rates between two study groups undergoing tonsillectomy in 2012 and in 2015. Furthermore, genetic alterations in tonsils harboring high-risk HPV infection were analyzed using a cancer gene panel.

## Patients and methods

### Study design

The study involved a total of 477 patients (median age 20.3, range 1.7–69.5 years) undergoing tonsillectomy because of tonsillar hypertrophy or chronic tonsillitis in Helsinki University Hospital, Department of Otorhinolaryngology – Head and Neck Surgery. Of the 477 patients, 237 (49.7%) were recruited consecutively between August and December 2012 (Group A), and 240 (50.3%) between April and September 2015 (Group B). During both study periods the patients were recruited in a similar consecutive manner on the day of operation. Collecting samples in two separate time periods enabled us to evaluate whether the prevalence of tonsillar high-risk HPV infection remained stable from 2012 to 2015 in Helsinki capital area, comprising a population of roughly 1 million. Demographics of the study participants are presented in Table 1. The Ethics Committee of Surgery in the Hospital District of Helsinki and Uusimaa approved the study protocol, and all patients gave their informed written consent.

### Collection of samples

One tonsil from each patient was individually processed for study purposes using sterile equipment. One half was fixed, embedded in

**Table 1**

Demographic data and tonsillar high-risk HPV status in patients undergoing tonsillectomy in 2012 (Group A) and in 2015 (Group B).

Parameter	Group A n (%)	Group B n (%)	Total N (%)
Number of patients	237 (49.7)	240 (50.3)	477 (100)
Gender			
Male	92 (39)	90 (38)	182 (38)
Female	145 (61)	150 (63)	295 (62)
Age distribution (y)			
< 7 Preschool children	42 (18)	63 (26)	105 (22)
7–16 School children	45 (19)	36 (15)	81 (17)
17–25 Young adults	65 (27)	66 (28)	131 (27)
26–40 Midlife adults	63 (27)	56 (23)	119 (25)
> 41 Older adults	22 (9)	19 (8)	41 (9)
HPV vaccination			
Yes	6 (3)	20 (8)	26 (5)
No	231 (97)	220 (92)	451 (95)
Smoking			
Never	153 (65)	160 (67)	313 (66)
Previous smoker			
< 20 pack-years	33 (14)	24 (10)	57 (12)
Previous smoker			
Over 20 pack-years	0 (0)	1 (0.4)	1 (0.2)
Current smoker			
< 20 pack-years	48 (20)	51 (21)	99 (21)
Current smoker			
Over 20 pack-years	3 (1)	4 (2)	7 (1)
High-risk HPV			
Negative	235 (99.2)	237 (98.8)	472 (99.0)
Positive	2 (0.8)	3 (1.3)	5 (1.0)

paraffin and examined using standard haematoxylin eosin staining sections to confirm benign non-neoplastic tonsillar histology without dysplasia. The other half was divided into small pieces and stored fresh either in Eppendorf tubes or phosphate-buffered saline at  $-80^{\circ}\text{C}$  until analyses. Peripheral blood samples were drawn from each patient to obtain genomic DNA from isolated blood leukocytes.

### Tonsillar HPV PCR analyses

A clinical diagnostic laboratory in Tartu, Estonia (Quattromed HTI, currently merged with Synlab Lab Services corporation) analyzed all tonsillar fresh tissue specimens for high-risk HPV. Each specimen was suspended in a lysis buffer containing 200  $\mu\text{l}$  Magna Pure Bacteria Lysis Buffer (Roche diagnostics, GmbH Mannheim, Germany) and 20  $\mu\text{l}$  of 20 mg/ml Proteinase K (Thermo Scientific, MA, USA) and incubated for 24 h at  $+37^{\circ}\text{C}$ . DNA extraction was completed with MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche diagnostics, GmbH Mannheim, Germany). Ten  $\mu\text{l}$  of the extracted DNA was used in PCR amplification. In each sample, beta-globin gene amplification confirmed DNA integrity. Luminex xMAP platform was used to detect 16 different high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 82. The assay comprised 12 pairs of primers of which four pairs are shared with different genotypes. One  $\mu\text{l}$  of the PCR product from each specimen was mixed together with Luminex beads, followed by hybridization at  $95^{\circ}\text{C}$  for five minutes, and thereafter cooling down to  $55^{\circ}\text{C}$  for 15 min to hybridize single stranded DNA products to HPV type specific oligonucleotides. Luminex MAGPIX instrument was used to detect high-risk HPV positivity.

Extracted DNA from Group A samples also underwent HPV 16 specific PCR analyses. Each 25  $\mu\text{l}$  reaction contained 1x GeneAmp<sup>®</sup> PCR Buffer I containing 15 mM  $\text{MgCl}_2$  (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 mM of each dNTP, 0.2  $\mu\text{M}$  HPV16.S2 forward primer (sequence: CAA CAA ACC GTT GTG TGA TT), 0.2  $\mu\text{M}$  HPV.CTC reverse primer (sequence: ATC TAT TTC ATC CTC CTC CTC T), 0.625 U of AmpliTaq Gold<sup>®</sup> DNA Polymerase (Thermo Fisher Scientific) and 25 ng

**Table 2**

Characteristics of 5 patients with tonsillar high-risk HPV infection.

Patient ID	Age (y)	Gender	HPV type	p16 IHC epithelium	Smoking	Indication for tonsillectomy
9	20	Male	52	+++	Never	Chronic tonsillitis
239	14	Male	16	+++	Never	Hypertrophy
429	21	Male	66	+++	Current smoker < 10 pack-years	Chronic tonsillitis
452	20	Female	16	+	Previous smoker < 10 pack-years	Chronic tonsillitis
487	20	Female	52 and 68	+++	Never	Chronic tonsillitis

Abbreviations: HPV, human papillomavirus; IHC, immunohistochemistry; +, weak staining; + + +, strong staining.

of the extracted DNA. The reactions were amplified in DNA Engine Tetrad® 2 Peltier Thermal Cycler (BioRad, Hercules, CA, USA) using the following conditions: Denaturation in 95 °C for 10 min followed by 40 cycles of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C, after which the reactions were cooled down to 4 °C.

#### Fluorescence in situ hybridization (FISH)

All specimens harboring high-risk HPV, detected by PCR, were subsequently analyzed by FISH. Positive controls included hybridizations on HPV 16 positive tissue from human uterine cervical intraepithelial neoplasia lesions. Negative controls were nonmalignant tonsillar specimens without HPV 16, as determined by PCR. HPV FISH was performed on 4 µm-thick tissue sections as described previously [3,4]. Briefly, sections were pretreated pretreated with 85% formic acid/0.3% H<sub>2</sub>O<sub>2</sub>, 1 M NaSCN and 4 mg/ml pepsine and hybridized with a digoxigenin-labeled HPV 16-specific probe (PanPath, Budel, The Netherlands) according to the manufacturer's instructions. After stringent washing in 50% formamide, 2xSSC, pH 7.0 at 42 °C (two times 5 min), probe hybridization was visualized by subsequent incubations with mouse anti-digoxin (Sigma), peroxidase-conjugated rabbit anti-mouse IgG, peroxidase-conjugated swine anti-rabbit IgG (both DAKO A/S) and finally rhodamine-labeled tyramide. Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenyl indole (DAPI; Sigma; 0.2 g/ml). The slides were then examined with the Leica DM5000B microscope with appropriate fluorochrome filter sets.

Evaluation of nuclear hybridization signals was performed by two investigators (AH and EJMS) according to previously described criteria [3,4]: diffuse, dotted-like signals indicate episomal HPV DNA and nuclear punctate signals were considered to indicate integrated HPV DNA.

#### p16<sup>INK4A</sup> immunohistochemistry

Tonsillar specimens from high-risk HPV positive patients and from their age- and gender matched controls were subjected to immunostaining using p16<sup>INK4A</sup> CINtec ready-to-use antibody (CINtec® Histology Kit, Roche, Germany). Study pathologist (JH) carefully evaluated the stained sections, blinded to the HPV-status. Immunostaining for p16<sup>INK4A</sup> was graded as negative, weak, moderate, or strong. Immunostaining for epithelial cells and germinal center dendritic cells was analyzed separately.

#### Detection of cancer gene mutations in patients harboring tonsillar high-risk HPV

A total of 10 tonsillar specimens, including five from patients harboring high-risk HPV infection and five from their age- and gender matched controls, were analyzed at Functional Genomics Unit (FuGU), Biomedicum Helsinki. To examine whether similar cancer gene mutations were present in tonsils and leukocytes, peripheral blood leukocytes from five high-risk HPV positive patients and two HPV negative

controls underwent similar analyses. DNA was extracted using NucleoSpin kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendation. The samples were sequenced with Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) in one lane/run using Roche Nimblegen SeqCap EZ, Comprehensive Cancer Design panel (Roche Nimblegen, Basel, Switzerland). The target size of the panel is 4 Mb and it targets 578 genes involved in different types of cancers. Sequencing was performed as paired end sequencing for read length 75 bp (PE75 or 2X75). The sequencing coverage across targeted bases was 200x and the target bases above 20x coverage were included in the analyses. The cut-off value for sequence variants was 2.5%

## Results

### Tonsillar high-risk HPV prevalence

Tonsillar high-risk HPV was detected by Luminex in 2 of 237 (0.8%) patients in group A, and in 3 of 240 (1.3%) patients in group B. HPV 16 specific PCR analyses found no additional positive samples in group A. Of five HPV positive patients, two males and two females were young adults, and one male was 14 years old. None of them had been vaccinated against HPV. Two patients were positive for HPV 16 and one for HPV 52. Two patients presented with weak HPV positivity: one for HPV 52, and the other for both HPV 52 and HPV 66. One patient with high-risk tonsillar HPV infection, a 20-year-old female (patient 452), had recurrent migraine type headaches, while all others presented with insignificant medical history. Clinical data on HPV positive patients are presented in Table 2.

### Fluorescence in situ hybridization (FISH)

Tonsillar specimens that were PCR positive for HPV 16 were also positive in HPV 16 specific FISH analyses, with episomal appearance (Fig. 1). Specimens presenting with HPV 52 and HPV 66 in PCR analyses were FISH negative.

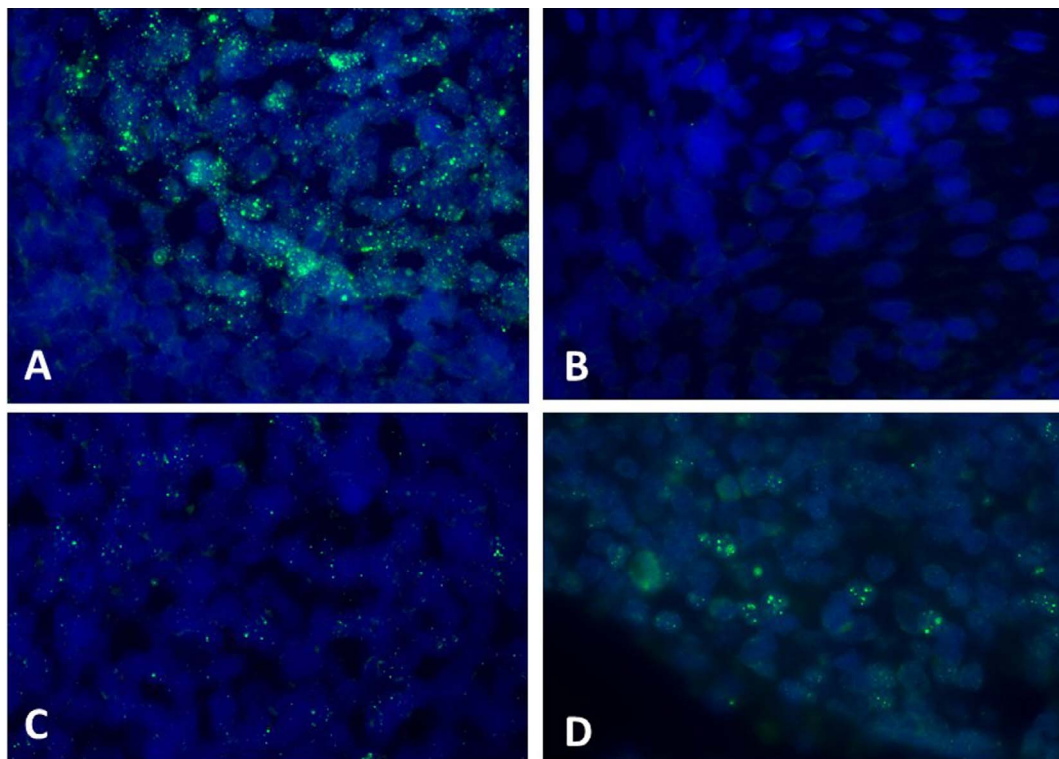
### p16<sup>INK4A</sup> immunostaining

All HPV positive tonsillar specimens and their matched controls were positive for p16<sup>INK4A</sup>. Immunohistochemical staining for p16<sup>INK4A</sup> in the crypt epithelium was strong in 4 of 5 (80%) specimens harboring high-risk HPV, whereas all HPV negative control specimens presented with either moderate or weak immunostaining. Germinal center dendritic cells presented with moderate p16<sup>INK4A</sup> staining in two specimens harboring HPV, while three HPV positive specimens and all controls presented with weak staining.

### Cancer gene mutations in tonsils harboring high-risk HPV

To explore mutations potentially associated with high-risk HPV infection, we considered mutations that were present only in high-risk HPV positive tonsils, but not in the corresponding peripheral blood leukocytes of the same patients, or in HPV negative control tonsils. The





**Fig. 1.** Fluorescence in situ images A. Positive control from uterine cervical intraepithelial neoplasia lesion with episomal appearance. B. HPV negative tonsil. C HPV 16 positive tonsil (patient 452) with episomal appearance D. HPV 16 positive tonsil (patient 239) with episomal appearance.

**Table 3**

Specific heterozygous, damaging mutations only present in  $\geq 2$  HPV positive tonsillar specimens, but absent in the patient leukocytes and in tonsillar specimens from HPV negative controls.

Gene	Patients (ID)	Variant	Chromosome	Coordinate	Exonic	Percentage of altered reads
<i>BAGE2</i>	429, 452	A > A/G	21	11029721	Yes	22
<i>BAGE2</i>	429, 452	A > A/G	21	11047334	No	44
<i>BCL10</i>	9, 429	C > C/CA, CAA > CAA/C	1	85732559	Yes	38
<i>BCL10</i>	9, 452	G > G/A	1	85732582	Yes	55
<i>CDKN2B-AS1,CDKN2B</i>	9, 429	A > A/G	9	22005920	Yes	27
<i>COL1A1</i>	9, 452, 487	C > C/CA, CA > CA/C, CAA > CAA/C	17	48261836	Yes	39
<i>EP300</i>	239, 487	A > A/C	22	41575892	Yes	34
<i>ETV6</i>	239, 487	T > T/TA	12	11905742	No	20
<i>FOXP1</i>	9, 239	C > C/CT	3	71007462	Yes	17
<i>IKZF1</i>	9, 429	G > G/GT	7	50444181	No	17
<i>MAF</i>	9, 239	AAC > AAC/A	16	79632187	Yes	11
<i>MAP2K2</i>	9, 429	G > G/A	19	4097234	No	22
<i>NF1</i>	429, 452	C > C/CT, CT > CT/C	17	29575849	No	19
<i>PIK3CA</i>	429, 487	C > C/CT, CT > CT/C	3	178917762	No	37
<i>PTCH1</i>	9, 239, 429	GAA > GAA/G, GA > GA/G	9	98245692	No	41
<i>RB1</i>	9, 452	G > G/T	13	49039122	Yes	21
<i>RUNX1</i>	452, 487	C > C/CT, CT > CT/C	21	36265323	No	26
<i>SEPT6</i>	429, 487	T > T/TA	X	118752506	Yes	19
<i>STIL</i>	9, 487	T > T/G	1	47749582	No	63
<i>TPR</i>	452, 487	TA > TA/T, T > T/TA	1	186300535	No	18
<i>WDR36</i>	9, 452	GT > GT/G	5	110463621	Yes	46

specific mutations present in at least two HPV positive tonsillar specimens ( $n = 21$ ) are presented in Table 3. These included *EP300*, *NF1*, *PIK3CA*, and *RB1* which, according to the previous studies, have shown enrichment of mutations in HPV positive head and neck SCC [18]. All exonic mutations presented in Table 3 were located either in the 3' untranslated region (UTR) or in splice sites. Therefore, none of the mutations resulted in amino acid substitutions. Mutations were not detected in *MLL3*, *FGFR2/3*, *NOTCH1*, *KRAS*, or in *FBXW7*, which are considered to play a significant role in HPV-associated head and neck SCC. Some genes that may be relevant in HPV-associated carcinogenesis (*DDX3X*, *ASXL3*, *ZNF750*, *TTN*, and *SYNE1*) were not included in the

Nimblegen SeqCap EZ Comprehensive Cancer Design panel. One male patient (patient 239) presented with a homologous mutation in *SSX1* gene, which is associated with an increased risk for synovial sarcoma [20]. This mutation was detected in both tonsillar DNA and peripheral blood leukocytes, and it is not likely to be associated with tonsillar HPV infection.

We also analyzed mutations that were present in two HPV negative tonsils, but not in the corresponding control leukocytes. These included mutations in *KRAS*, *FBXW7*, *EP300*, *RB1*, and *NF1*. Thus, also HPV negative tonsils harbored mutations in genes that, according to the literature, are considered relevant in HPV carcinogenesis. However,

also HPV positive tonsils frequently harbored mutations in these specific locations.

## Discussion

In this study, only 5 out of 477 (1%) patients undergoing tonsillectomy because of chronic tonsillitis or tonsillar hypertrophy harbored high-risk HPV in tonsils, and the prevalence was similar in 2012 and 2015. In a previous study conducted at Helsinki University Hospital, benign tonsillar specimens were collected consecutively from tonsillectomy patients in 2001 and 2002. In that study, tonsillar HPV was detected from fresh tissue specimens using consensus-primer PCR in 6.3% (13 of 206) of patients. Only oncogenic HPV 16 was detected, and 11 of the 13 HPV positive cases presented either in children or in young adults aged < 26 years [21].

The prevalence of high-risk HPV infections is likely to decrease in Finland because bivalent HPV vaccination was included in the Finnish national vaccination program in November 2013. We showed, however, that the prevalence of tonsillar HPV infection decreased already between 2002 and 2012. The difference may be partly explained by different methodologies, but otherwise the reason for this is unclear. HPV etiology has not been implicated in tonsillar hypertrophy or chronic tonsillitis. Therefore, HPV prevalence among patients undergoing tonsillectomy is likely to reflect the overall prevalence in the general population.

The annual age-adjusted incidence of oropharyngeal cancer in the Finnish population, per 100,000, is 2.3 in males and 0.8 in females. Even if the prevalence of tonsillar high-risk HPV infection is  $\leq 1\%$  (1000 per 100,000 or less) a small fraction of the infected individuals will ultimately develop oropharyngeal HPV-associated cancer. In the present study, altogether 160 of our 477 patients (34%) were midlife adults or older. Interestingly, HPV was detected only in younger subjects, and not in the older age groups where HPV-associated premalignant findings might be expected.

The incidence of cervical HPV infection peaks soon after sexual debut at 20–24 years of age, and decreases thereafter [22]. The incidence of oral HPV infection appears to be more stable through all age groups [23]. Oral and oropharyngeal HPV infections are likely to have both sexual and non-sexual routes of transmission [24]. A high number of lifetime sexual partners is associated with an increased risk for oropharyngeal SCC [25]. However, the association between sexual behavior and presence of HPV in benign tonsils is unclear. In the present study, the majority (4 of 5) of the infected were young adults, who possibly harbored recent sexually transmitted infections. Previous studies, however, have found tonsillar HPV infections also in children [21,26]. HPV transmission can occur already before birth from mother to child, or horizontally during infancy and childhood, possibly through saliva or other routes [24].

Our study is consistent with the previous literature in that the prevalence of tonsillar high-risk HPV infection in cancer-free patients is low. Palmer and colleagues found HPV DNA neither in a material of 3377 formalin-fixed paraffin-embedded tonsils, nor in 511 homogenized fresh tonsil specimens, collected between 2004 and 2008 in the UK. The authors concluded that, contrary to cervical high-risk HPV infections, a high proportion of tonsillar high-risk HPV infections may progress to cancer [7]. Other previous studies, some of which have included only either children or adults, have reported tonsillar HPV prevalence rates from 0 to 12% [27–34]. Kim et al. found HPV in 8 of 69 (12%) tonsillar samples collected from patients with chronic tonsillitis. In that study, real-time PCR was used to analyze whether HPV DNA was episomal or integrated. E2/E6 ratio in three HPV 16 positive tonsils showed purely episomal forms [29].

Previous studies have not reported HPV positivity in benign tonsillar specimens with in situ hybridization. Klingenberg et al. found high-risk HPV by PCR in 2 of 195 (1%) non-malignant tonsils. However FISH analysis was unable to detect oncogenic HPV 16 and 18 in the

PCR-positive specimens [31].

Possibly due to its porous structure and the lack of structural integrity, tonsillar reticulated squamous epithelium is more vulnerable to HPV infection than other anatomical sites of the upper aerodigestive tract. In HPV positive tonsillar SCC, patients typically have neck lymph node metastases at presentation, although the primary tumor is often small [4,6]. Rietbergen et al. showed that in specimens from 20 patients with HPV positive oropharyngeal SCC, tumor-free resection margins were all negative for HPV16 E6 mRNA [35]. Franceschi et al. showed that deep brushing of 200 non-pediatric tonsils, three of which (1.5%) were HPV positive, was an unsatisfactory method for non-invasive detection of tonsillar precancerous lesions; the cytology slides were of poor quality and those suspected of having atypical cells did not show dysplasia in the formalin-fixed paraffin-embedded tissue blocks [36]. The low detection rate of HPV infections in benign tonsils, and the difficulty in identifying premalignant lesions analogous to cervical intraepithelial neoplasia, may be explained by the HPV infection being restricted to an extremely small area, possibly deep in the tonsillar crypts. However, FISH analyses in a study by Mooren et al. showed that dysplastic epithelium adjacent to HPV positive tonsillar SCC had positive punctate HPV pattern, indicating viral integration [37]. Dysplastic tonsillar epithelium with integrated high-risk HPV may be a precursor lesion in tonsillar carcinogenesis. In the present study, FISH analyses showed episomal appearance of HPV 16 without dysplasia. To our knowledge, this is the first study reporting high-risk HPV positivity by FISH in benign tonsils.

In this study, we chose a comprehensive cancer gene panel to explore genetic alterations associated with high-risk HPV infection. High-risk HPV positive tonsils harbored mutations in *EP300*, *NF1*, *PIK3CA*, *RICTOR*, and *RB1*. In a recent study by Chung et al. these five genes were among the 30 most commonly altered genes in HPV positive head and neck SCC. The study evaluated genomic alterations of head and neck SCC using FFPE specimens which included 84 from HPV positive tumors [38]. The results were in line with the previous studies in that *PIK3CA* was the most commonly altered gene in HPV positive tumors [15]. Whether the mutations in *EP300*, *NF1*, *PIK3CA*, *RICTOR*, and *RB1* are associated with high-risk HPV infections cannot be verified in our material since the number of HPV positive samples was low. Mutations in *NF1* and *PIK3CA* were intronic, and the relevance of these specific mutations in tonsillar SCC is unclear. However, malignancy-driving mutations can occur in all genetic elements outside the coding region, also in enhancer, silencer, insulator as well as in 5'UTR and 3'UTR [39]. In our material, also HPV negative tonsils harbored mutations in genes that are considered relevant in HPV carcinogenesis. These specific locations frequently harbored mutations also in HPV positive tonsils. Potentially, some mutations do not promote tumorigenesis in HPV negative tissue where p53 and Rb function normally, but are significant with high-risk HPV infection when the functions of p53 and Rb are inactivated. In the present study, a low cut-off value of 2.5% was set for sequence variants, since only a small proportion of benign cells may harbor HPV-associated genetic alterations. Therefore, mutations present at low numbers could be missed when using a high cut-off value.

In four of five tonsils with high-risk HPV infection p16<sup>INK4A</sup> immunostaining was strong. In tonsillar SCC p16<sup>INK4A</sup> is used as a surrogate marker for HPV-association [40]. Our findings are in line with the previous studies in that HPV negative benign tonsils often show positive p16<sup>INK4A</sup> immunostaining [31]. Although p16<sup>INK4A</sup> immunostaining was more pronounced in our high-risk HPV positive specimens, and also observed in germinal center dendritic cells, the presence or specific location of high-risk HPV cannot be analyzed by using p16<sup>INK4A</sup> immunohistochemistry.

## Conclusion

The prevalence of high-risk HPV in nonmalignant tonsils was only 1%. FISH analysis showed that the appearance of HPV 16 was episomal.

High-risk HPV positive tonsils harbored mutations in genes that are commonly altered in HPV-associated head and neck SCC: *EP300*, *NF1*, *PIK3CA*, *RICTOR*, and *RB1*. However, due to the low rate of HPV positive tonsils and the absence of tonsillar precancerous lesions, the involvement of these mutations in HPV-associated carcinogenesis requires further research.

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None declared.

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